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Interaction of the mitochondrial NADH-ubiquinone reductase with rotenone as related to the enzyme active/inactive transition [1]

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Abstract

The interaction of rotenone with active ('pulsed') and thermally de-activated ('resting') membrane-bound Complex I (Kotlyar, A.B. and Vinogradov, A.D. (1990) Biochim. Biophys. Acta 1019, 151–158) as revealed by inhibition of NADH-ubiquinone- and ubiquinol-NAD^+ reductase activities was studied. $K_i = 1 \cdot 10^{-9}$ M, $k_{on} = 5 \cdot 10^7$ M^{-1} min^{-1} and $k_{off} = 0.02$ min^{-1} (inhibitory effect of rotenone on NADH oxidation) and $K_i = 2 \cdot 10^{-8}$ M (inhibition of reverse electron transfer) were determined for pulsed enzyme. The equilibrium between de-activated and active enzyme is reached ($K \sim 100$) after the slow strongly temperature-dependent de-activation process has completed. Rotenone partially prevents and reverses the enzyme de-activation. About two order of magnitude difference in affinity of rotenone to the active and de-activated forms of the enzyme was demonstrated. The strong difference in rotenone sensitivity of the direct and reverse reactions can not be accounted for $\Delta\bar{\mu}_{H^+}$-dependence of rotenone binding. We propose that two rotenone-specific inhibitory sites exist in Complex I: one is involved in NADH oxidation by ubiquinone and the other is operating in ubiquinol-NAD^+ reductase reaction. The affinities of rotenone for both sites are strongly altered upon the slow enzyme active/inactive transition.

Keywords: NADH-ubiquinone reductase; Complex I; Rotenone; Tight-binding inhibitor; Respiratory chain; (Bovine heart mitochondrion)

1. Introduction

NADH-ubiquinone oxidoreductase (EC 1.6.99.3), operationally termed Complex I [1–3] is an extraordinarily complex proton motive device of the mitochondrial respiratory chain. The enzyme comprises of more than 40 different polypeptides that are encoded both in the nuclear and mitochondrial genomes [4]. FMN [5], several iron-sulfur clusters [6] and bound ubiquinone [1,7] participate in the reversible electron transfer from NADH to bulk ubiquinone coupled

Abbreviations: SMP, submitochondrial particles; BSA, bovine serum albumin; Q, natural ubiquinone (Q_{10}).

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[1] Dedicated to the memory of our colleague Dr. Vladimir D. Sled, a devoted scholar of Complex I who tragically deceased on October 31, 1996.

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with the proton translocation across the mitochondrial inner membrane. Despite a great progress in the structural studies of the mammalian [3,4], *Neurospora crassa* [8] enzymes and their simpler bacterial counterparts [9,10], neither the sequence of electron transfer between the intrinsic enzyme redox components nor the mechanism of proton translocation are known.

The specific inhibitors have proved to be a useful tool for studies on the mechanism of electron transfer and proton translocation in the respiratory chain. Rotenone [11–15] and piericidin [16] are the most powerful specific inhibitors of Complex I which are widely used for dissection of the $\Delta\overline{\mu}_{H^+}$-generating electron transfer reactions in NADH-ubiquinone region of the respiratory chain. It is generally accepted that rotenone blocks the electron transfer between the Complex I-associated iron-sulfur clusters and ubiquinone pool [15]. More specifically, rotenone prevents a formation of the Complex I-associated ubisemiquinone species [7,17] thus most likely it inhibits the electron transfer between iron-sulfur center N-2 and tightly bound ubiquinone. Ernster et al. [12] demonstrated no reactivation of NADH oxidase by mixing together rotenone- and antimycin-treated particles as it might be expected for the specific irreversible inhibition. Restoration of NADH oxidase in the combination of rotenone-treated and ether-extracted particles has been claimed in other studies [18]. The specificity and stoichiometry of rotenone binding was the subject of the detailed studies of Singer's group [14,19–21]. They found that rotenone and piericidin bind to the specific inhibitory site at NADH-ubiquinone junction and to some other hydrophobic components in the submitochondrial particles [14,19]. BSA readily dissociates rotenone from the 'unspecific' sites leaving the enzyme in the inhibited form which tends to reactivate only after several repeated washing with albumin [14]. The inhibitory rotenone was extracted from the particles in its original chemically unmodified form by acetone, however addition of unlabelled rotenone to the particles previously labelled with [^{14}C]rotenone did not result in displacement of the bound label [14]. Photolabelling of Complex I with arylazido-analogue [22] and [^{3}H]dihydrorotenone [23] showed that 33 kDa mitochondrially encoded subunit is involved and evidence have been presented that binding of rotenone results in gross conformational changes of the enzyme moving several of the subunits away from each other [24]. The binding and inhibition by rotenone is believed to be non-competitive with ubiquinone [25–29]. Despite extensive studies on rotenone inhibition several obvious questions concerning the enzyme-inhibitor interaction either have not been answered or still are controversial. For example, we were unable to find in the literature K_i values for inhibition of any rotenone-sensitive reaction catalyzed by the membrane-bound or isolated preparations of Complex I: the I_{50} values scattered in the literature and expressed as mol of the inhibitor per mg of protein are almost meaningless when reversibility of the inhibition, its time-dependency and the stoichiometry of the specific binding are ill-defined.

In addition to the extremal structural complexity of the rotenone-sensitive enzyme the latter demonstrates functional heterogeneity. Following the pioneering observations of Tyler et al. [30] we have provide evidence that the membrane-bound [31,32] and isolated [33] Complex I exist in the 'resting' (de-activated) or 'pulsed' (active) forms. The slow interconversion between those forms depends on several factors [34] and there are strong reasons to believe that the NADH-ubiquinone reductase (in submitochondrial particles or as purified Complex I) as prepared is always a mixture of pulsed and resting forms. It was rather unexpectedly shown that thermally de-activated Complex I within tightly coupled submitochondrial particles becomes partially reactivated (as revealed by the energy-dependent NAD^+ reduction) after incubation in the presence of rotenone and subsequent removal of the inhibitor [31]. Further studies on Complex I-rotenone interaction may provide new information on the functional properties of NADH-ubiquinone reductase. The studies reported here were concerned with two mutually related subjects: one was to quantitate the kinetics and thermodynamics of the rotenone-induced inhibition; the second part was aimed to further examination of the slow active/inactive enzyme transition.

2. Materials and methods

Coupled submitochondrial particles (SMP) were prepared as described previously [31]. Protein content

was determined with biuret reagent [35]. Two types of the preparations were used.

2.1. De-activated particles

SMP (5 mg/ml) were suspended in 0.25 M sucrose, 50 mM Tris/Cl^-, 0.2 mM EDTA, 2 mM malonate (potassium salts, pH 8.0) and oligomycin (0.5 μg/mg of protein) were added. The mixture was incubated for 1 h at 30°C, cooled and stored during the experiments at room temperature ($\sim$ 20°C). De-activated particles showed prominent lag-phase in their NADH oxidase or succinate-supported NAD^+ reduction and both activities were strongly inhibited by Ca^{2+} or Mg^{2+} [32].

2.2. Activated particles

SMP (5 mg/ml) suspended and treated with oligomycin as described before were incubated aerobically with a constant stirring in the presence of 1 mM NADPH for 20 min. The suspension was passed through the Sephadex column as described [17] (bovine serum albumin was excluded), cooled and stored during the experiments at 0°C. Occasionally, when very diluted suspensions were used (less than 50 μg/ml) the activation was reached by the addition of 5 μM NADH to the assay cuvettes and incubation for a time period needed for complete oxidation of NADH (followed spectrophotometrically). The activated particles showed no lag-phase in their NADH oxidase or succinate-supported NAD^+ reduction and both activities were insensitive to Ca^{2+} or Mg^{2+} [32].

The enzymatic activities were assayed at 25°C in the standard reaction mixture containing 0.25 M sucrose, 50 mM Tris/Cl^-, 0.2 mM EDTA (pH 8.0). Other additions and the details of the experiments are described in the legends to the figures.

Rotenone (stock solution) was dissolved in dimethylsulfoxide. The solubility of rotenone in different solvents at 20°C was determined as follows. The solid dry rotenone was stirred in dark in water, octane, octanol and cyclohexane (spectrally pure) for 4 hours. The suspensions were centrifuged and the U.V. spectra of clear supernatants were recorded. The concentrations of saturated rotenone solutions were calculated using $E_{292}^{\mathrm{mM}} = 17$. The latter value was determined using analytically prepared rotenone solution in ethanol. The solubilities determined were: water - 1.6 μM; octane - 0.3 mM; octanol - 6.2 mM; cyclohexane - 1.2 mM. The organic solvent/water partition coefficients calculated as the solubilities ratio were: octane/water: $\sim$ 200; octanol/water: $\sim$ 4000; cyclohexane/water: $\sim$ 800. Almost the same partition coefficients for octane/water and cyclohexane/water were determined by measuring rotenone concentrations after shaking and separation of two-phase systems. The final concentration of rotenone in all the experiments never exceeded the solubility limit.

Rotenone, NADH, NAD^+, malonate, succinate, oligomycin, gramicidin D, EDTA, antimycin A, bovine serum albumin (BSA, fraction V) were from Sigma (USA); Tris was from Serva (FRG). Piericidin was a kind gift from Dr. A.B. Kotlyar (Department of Biochemistry, Tel-Aviv University, Israel). Other chemicals were of the highest quality commercially available. All calculations and curve fittings were performed using GIM Version 2 computer program (A. Drachev Copyright).

3. Results

3.1. Kinetics of the active Complex I inhibition by rotenone

Trivial interpretation of the enzyme-inhibitor interaction is possible when the reversible inhibition is studied under the steady-state conditions where the concentration of a single enzyme specie is much less that of the inhibitor and when all the equilibria are established instantly within the time scale of an assay. Those conditions are definitely not hold for inhibition by rotenone. The possible difficulties in clear interpretation of the inhibition mechanism may result from the heterogeneity of the rotenone-sensitive Complex I preparations [31–34]. Since the conditions for the reversible interconversion between resting and pulsed enzyme and some diagnostic tools to measure a fraction of the active enzyme have been established [32,34], it was of interest to measure the parameters of functionally homogeneous Complex I-rotenone interaction. Fig. 1 depicts the inhibition time-course for the pulsed particles under the condi-

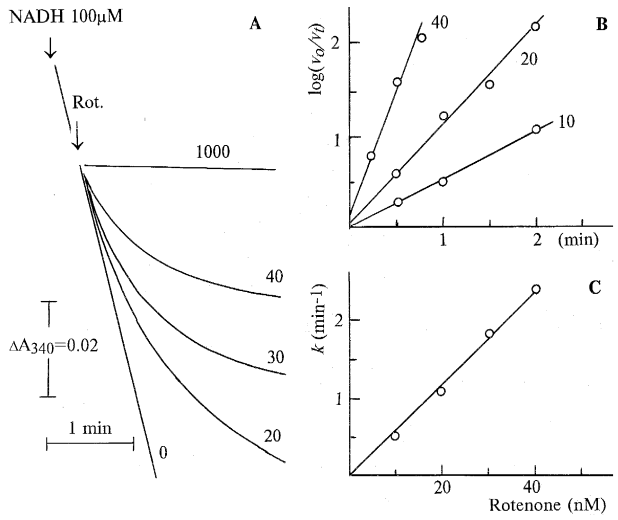

Fig. 1. (Panel A) Time-course of NADH oxidase inhibition by rotenone. SMP (12 μg/ml, approx. 2 nM Complex I) were placed in the standard reaction mixture and the enzyme was activated by the addition of 5 μM NADH (see Section 2). Gramicidin (0.04 μg/ml) was then added and the reaction was started by the addition of NADH where indicated. The numbers on the curves indicate the final concentration of rotenone (Rot.) expressed in nM. (Panel B) Semilogarithmic anamorphoses of the rate decrease at different rotenone concentrations. v_o, zero-order rate of NADH oxidation in the absence of rotenone; v_t, the 'instant' values of the rates approximated within 10 s time intervals. (Panel C) The dependence of apparent first-order inhibition rate constant on the concentration of rotenone.

tions where the inhibitor concentration was much higher than that of the enzyme. The inhibition was evidently time-dependent and it obeys first-order kinetics both as a function of time and the inhibitor concentration. The second-order rate constant of $5 \cdot 10^7$ M^{-1} min^{-1} was determined from the data shown in Fig. 1. The inhibition by 1 μM rotenone was instant within the time resolution limit as it might be expected from the second-order rate constant value.

When NADH oxidase activity was titrated by preincubation of the relatively concentrated suspension of SMP with rotenone the straight line was obtained (Fig. 2A). This is in accord with the earlier results of Lindahl and Öberg [11] and Ernster et al. [12] who have interpreted their data in terms of the irreversible inhibition. The amount of the rotenone-titrated component calculated by extrapolation of the straight line was 0.16 nmol/mg of protein. This is in a reasonable agreement with FMN content in submitochondrial particles [36,37] suggesting 1:1 stoichiometry for the NADH-ubiquinone reductase: inhibitory rotenone. Obviously, no clear cut border between reversible and irreversible inhibition exists and an appearance of an inhibition as either the linear hyperbolic or titration-type (as in Fig. 2A) curves depends on K_i value and the relative concentrations of an enzyme and inhibitor. Indeed, when the titration experiment was carried out in the diluted system a simple hyperbolic inhibition was evident (Fig. 2B). The hyperbolae shown in Fig. 2B (curve 1) fits the simple single site directed reversible inhibition with K_i value of 1 nM. Since Complex I is the proton-translocating enzyme and $\Delta\overline{\mu}_{H^+}$ generation is rotenone-sensitive it was of a considerable interest to find out whether $\Delta\overline{\mu}_{H^+}$ has any effect on the enzyme-inhibitor interaction. The results presented in Fig. 2B show that this is not the case. The K_i value and the second-order rate constant for a formation of the enzyme-inhibitor complex give the calculated first-order dissociation rate constant of 0.05 min^{-1}. The latter was directly estimated in the experiments shown in Fig. 3. Reasonable coincidence between the calculated (0.05 min^{-1}) and experimental (0.02 min^{-1}) values was obtained. The experiment shown in Fig. 3 provided an independent approach to support a simple model for a single-site directed reversible inhibition. It could be anticipated that the final level of the NADH oxidase activity reached after equilibration followed the dilution of the stoichiometric enzyme-rotenone complex would follow approximately the square root dependence on the added complex concentration. When the 'stoichiometric' enzyme-rotenone complex (0.5 μM) was diluted 100- and 1000-fold and left to equilibrate for 2 hours in the medium containing no BSA as shown in Fig. 3, the final levels of the activity recovered were 30% and 63% of the original uninhibited level, whereas the theoretical calculations assuming K_i of 1 nM give the values of 36 and 72, respectively.

3.2. Kinetics of the de-activated Complex I interaction with rotenone

Fig. 4 illustrates qualitative difference in the interaction of rotenone with active and de-activated Complex I. When the reaction was started by the simultaneous addition of NADH and rotenone to the pulsed enzyme the time dependent progressive inhibition

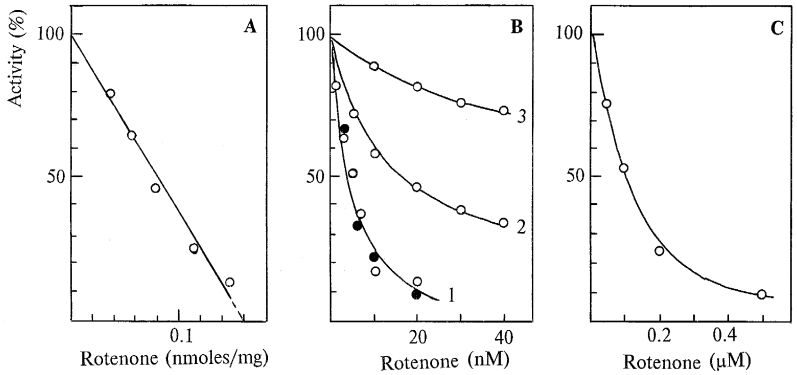

Fig. 2. Relative affinities of rotenone to the inhibitory site(s). (Panel A) Activated SMP (2.8 mg/ml, approx. 0.4 μM Complex I) were incubated in the standard reaction mixture for 20 min at 25°C and residual initial rate of NADH oxidation was measured as in Fig. 1. 100% correspond to the specific activity of 1.0 μmol/min per mg of protein. (Panel B) curve 1 (○), SMP (48 μg/ml, approx. 8 nM Complex I) were activated in the assay cuvette as in Fig. 1, and preincubated with rotenone in the presence of gramicidin and 10 mM malonate for 20 min at 25°C and the residual NADH oxidase activity was then measured; (●), the same as (○), except that preincubation with rotenone was made in the presence of 10 mM succinate (no gramicidin and malonate) to maintain $\Delta\mu_{H^+}$; 10 mM malonate and gramicidin were added simultaneously with 100 μM NADH to measure the residual activity. Curve 2, the reverse electron transfer activity. The preincubation was as described for curve 1 (○) except that no gramicidin and malonate were added and the residual rate of NAD^+ reduction was measured after simultaneous addition of 10 mM succinate and 1 mM NAD^+. 100% correspond to the specific activity of 0.2 μmol of NAD^+ reduced/min per mg of protein. Curve 3, de-activated SMP were preincubated with rotenone as described for curve 1 (○) and the residual steady-state NADH oxidase 1 min after 5-times dilution of the preincubation medium with the standard assay mixture containing 100 μM NADH and gramicidin was measured. The controls (100%) for each point correspond to the activity measured in the presence of the residual amounts of rotenone added with the preincubation medium to the assay system. The maximal inhibition by that residual rotenone did not exceed 10%. (Panel C) The same as Panel B, curve 3, except for enzyme concentration was 0.5 mg/ml and rotenone concentration range which was increased to show interaction of the inhibitor with de-activated enzyme. The activity was measured after 200-fold dilution into the assay mixture. All the continuos lines corresponds to the theoretical titration curves for the reversible single site inhibition with K_i values of 1 nM, 20 nM and 80 nM for the curves 1, 2 and 3, respectively.

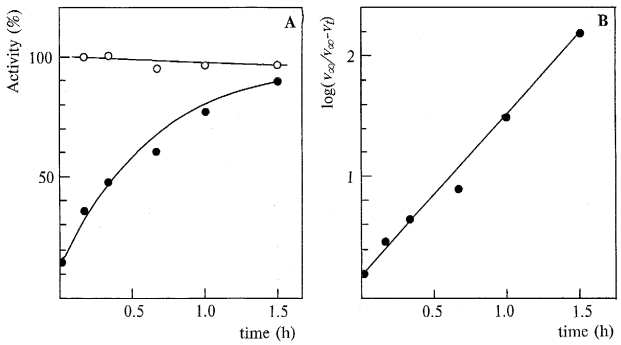

Fig. 3. Kinetics of NADH oxidase reactivation after dilution of the rotenone-inhibited enzyme. (Panel A) (●) active SMP (3 mg/ml, approx. 0.5 μM Complex I) were preincubated with 0.5 μM rotenone for 20 min at 20°C. The suspension was diluted 1000-times with the standard reaction mixture containing BSA (1 mg/ml). 2 ml of the diluted mixture were placed into the assay cuvette at the time indicated and NADH oxidase was measured as in Fig. 1. (○), v (no rotenone was added). (Panel B) Semilogarithmic anamorphose of the curve shown in Panel A; the first-order rate constant determined from the straight line is equal to 0.02 min^{-1}.

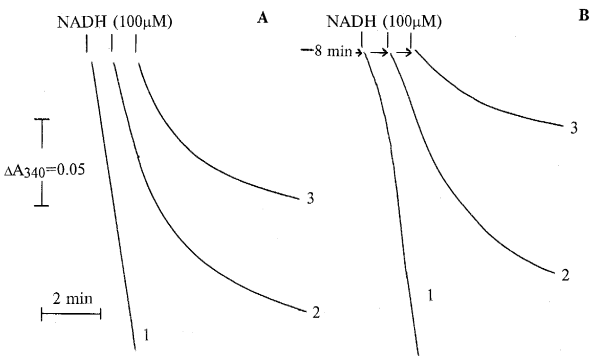

Fig. 4. Time-course of the active and de-activated enzymes inhibition by rotenone and piericidin. (A) SMP (25 μg/ml) were activated by the consumption of 5 μM NADH. Gramicidin was added and reaction was initiated by the simultaneous addition of NADH (curve 1, control) and rotenone (20 nM, curve 2) or piericidin (4.5 nM, curve 3). (B) de-activated SMP (25 μg/ml) were preincubated for 8 min with no inhibitors (curve 1) or in the presence of 20 nM rotenone (curve 2) or 4.5 nM piericidin (curve 3) and the reaction was started by the addition of NADH. Note short prominent lag-phase in the control assay (curve 1).

was observed (panel A, curve 2). However, when completely de-activated enzyme was preincubated with rotenone for a time period needed to reach about 80% inhibition of the active enzyme and the reaction was started by NADH, no inhibition was observed and the time needed to reach 80% inhibition was almost the same (panel B, curve 2). Interestingly, the same difference in the inhibition pattern was demonstrated for piericidin (curves 3). The results are in agreement with the earlier data of Burgos and Redfearn [13] and suggest that either rotenone does not interact with the de-activated enzyme or the inhibitor has much lower affinity to the latter. To solve this alternative the equilibrium titration experiments were carried out using de-activated enzyme and K_i value of 80 nM was determined (Fig. 2, panel B, curve 3 and panel C). That value and K_i for the active enzyme were independently verified in the experiments aimed to determine the residual rotenone concentration in the medium after incubation with active and de-activated enzyme preparation (Fig. 5). The experimental points closely correlate with the predicted values calculated assuming K_i value for active and de-activated enzyme preparations of 1 nM and 80 nM, respectively.

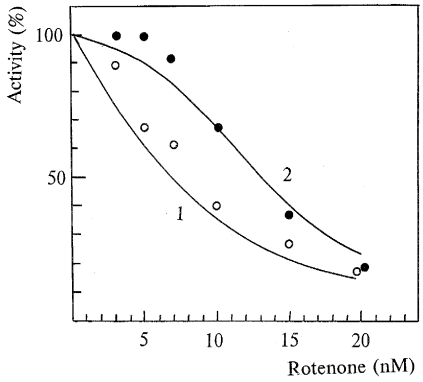

Fig. 5. Comparison of binding of rotenone with active and de-activated SMP. The samples of active and de-activated SMP (50 μg/ml, approx. 8 nM Complex I) were incubated in the standard mixture for 20 min at 20°C with different concentrations of rotenone indicated on abscissa. The suspensions were cooled and particles were separated by centrifugation at 4°C 1 h, 30000 $\times g$. No NADH oxidase activity was present in the supernatants. The amount of unbound rotenone in the supernatants was determined by their inhibitory effect on NADH oxidase activity of active SMP as follows. 1 ml samples of the supernatants were mixed with 1 ml of the assay mixture and NADH oxidase activities of active SMP (25 μg/ml, approx. 4 nM Complex I) were measured after 20 min preincubation at 25°C in the assay mixtures containing the supernatants with unbound rotenone. (●) Rotenone was preincubated with active SMP; (○) rotenone was preincubated with de-activated SMP. The continuos lines were theoretically calculated assuming K_i values for rotenone of 1 nM and 80 nM for active and de-activated SMP, respectively. The upward deflections of the experimental points most likely due to unspecific rotenone binding at the hydrophobic membrane phase (see partition coefficients for rotenone in Section 2 and Refs. [14,19]).

3.3. Slow active / inactive transition as an equilibrium process

In our previous publications on the active/inactive Complex I transition the thermally induced de-activation process was considered as an irreversible process, where the only specific pathway for reactivation includes at least a single slow enzyme turnover [31–34]. However, it has been noted that partial reactivation slowly occurred (as revealed by its catalytic activity in the energy-dependent reverse electron transfer) when thermally de-activated SMP were incubated with rotenone [31]. This unexpected finding have prompted us to get closer insight in the kinetics of the thermal de-activation. Fig. 6 depicts the time-course of the NADH oxidase and reverse electron transfer activities de-activation which appears as the first-order reactions. However, when the de-activation was followed for a longer time the residual rotenone-sensitive activities remain constant indicating that the process reached an equilibrium. The same equilibrium level of the residual activity was determined at 30, and 39°C, whereas the rate of de-activation was extremely temperature-dependent in accord with our previous reports [31,33].

3.4. Effect of rotenone on the active / inactive transition

It could be anticipated from the dramatic difference in the inhibitor affinity to the active and de-activated enzyme and from the equilibrium between those forms (Fig. 6), that rotenone should have profound effect on the equilibration between the active and de-actived forms of Complex I. In order to demonstrate and quantitate the activating effect of rotenone the following experimental approach was used. In accord with the data reported by Kotlyar and

Gutman [38] (but not in agreement with their interpretation, see Fig. 2 and Section 4) we found that sensitivity of the energy-dependent electron transfer to rotenone is considerably less than the NADH-ubiquinone reductase or NADH oxidase (Fig. 2). Moreover, inhibition of the reverse electron transfer was rapidly abolished in the presence of sufficient amounts of BSA. Thus, the active enzyme fraction could be measured in the reverse electron transfer assay in the presence of BSA. The results presented in Fig. 7 show that rotenone partially protects the enzyme against de-activation and partially reactivates the enzyme after the de-activation process has completed. It was noted (see Section 3.3) that the equilibrium between active and de-activated enzyme does not depend on temperature. Since the residual activity in the experiments depicted in Fig. 6 was only a small fraction of the full catalytic capacity of the enzyme an accuracy of that notion is not high. The temperature independence of the equilibrium was much more prominent in the experiments shown in Fig. 7 where the final level of the activity reached about 50% of the original value. It is evident that the protecting and reactivating effect of rotenone in the thermally induced de-activation is due to the significant difference in the affinity of the inhibitor to the different enzyme forms which are present as the slowly equilibrating mixture.

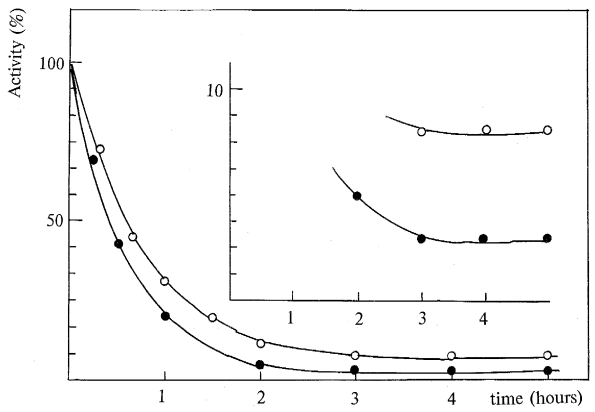

Fig. 6. Time-course of de-activation. Active SMP (2 mg/ml) were incubated at 30°C and the initial rates of uncoupled NADH oxidase in the presence of 15 mM $MgCl_2$ [32] (○) and rotenone-sensitive succinate-ferricyanide reductase (reverse electron transfer) in the presence of 10 mM succinate and 100 μM ferricyanide (●) were assayed. Insert: magnified ordinate at the time intervals indicated. The slight difference in the residual activities for (○) and (●) is due to incomplete inhibition of inactive → active transition during assay in the presence of Mg^{2+} [32], thus a precision of the kinetics and equilibria is much better for the reverse electron transfer assay. 100% correspond to the specific activities of 1.1 μmol/min per mg (○) and 0.5 μmol/min per mg (●).

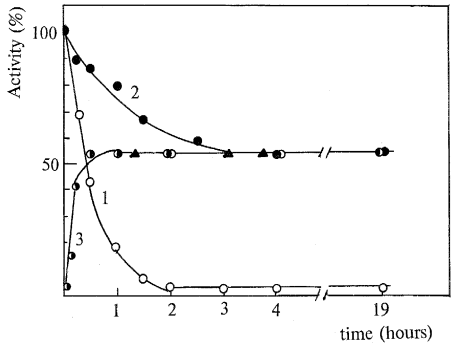

Fig. 7. Effect of rotenone on active/inactive transition. Active SMP (2 mg/ml) were incubated at 30°C or 39°C (▲) and the rates of rotenone-sensitive succinate-ferricyanide reductase (reverse electron transfer) were measured in the standard reaction mixture containing BSA (1 mg/ml) after 10 min incubation of SMP (20 μg/ml) to remove rotenone. Curve 1 (○), no rotenone was added; curve 2 (●), 2 μM rotenone was added; curve 3 (◑), 2 μM rotenone was added after de-activation in the absence of rotenone has completed. 100% correspond to the specific activity of 0.5 μmol/min per mg.

4. Discussion

Large number of the substances are known to inhibit NADH-ubiquinone reductase at the quinone junction site ([25,27,28,39–41] and references cited therein). Most, if not all of them act at the site which is the same or close to that where inhibitory rotenone binds. In recent years there have been significant advances in finding rotenone-like inhibitors [39–41] and it is commonly used practice to relate the effects of new inhibitors to those of rotenone. In light of the data reported here this approach may lead to erroneous conclusions and clear description of the inhibitory effect of rotenone in the simple text-book terms seemed desirable.

Since the original studies of Burgos and Redfearn [13] it is generally accepted that an 'unusual' feature of the inhibition by rotenone is the lag-period which occurs before the full effect in the inhibition becomes established. The kinetic and equilibrium constants we

have determined are characteristic for the tight-binding inhibitors [42]. We have previously experienced this type of inhibition in our studies of succinate dehydrogenase (EC 1.3.99.1)-oxaloacetate interaction [43] and several features of the tight binding kinetics worth to emphasize. When the affinity of an enzyme to an inhibitor is very high (K_i has the same order of magnitude as the concentration of an enzyme under the conditions used for assay, or less) both the association and dissociation reactions becomes very slow. The former is slow because the apparent first-order rate constant ($k_{on} \cdot [I]$) is small when very low concentration of an inhibitor is required for significant inhibition. The latter is slow because of intrinsic nature of tight binding. The second-order rate constant for inhibition of 'pulsed' Complex I ($5.5 \cdot 10^7$ M^{-1} min^{-1} at 25°C) is within the range for rapid enzyme-substrate (ligand) interaction with the upper limit set by the diffusion [44]. Thus bimolecular interaction of rotenone and Complex I is as rapid as that expected for the substrates (NADH and ubiquinone). It follows that the equilibration between Complex I, rotenone, substrate (quinone) and/or another inhibitor will be never reached within the time scale of the enzymatic assay and the type of inhibition would always appear as non-competitive independently of whether real competition between rotenone and a substance under investigation for the common binding site exists. Indeed, no competition between quinone acceptor and rotenone was found when assumption on equilibrium was implicitly applied for non-equilibrium conditions [27,28]. On the other hand, an apparent competition would only become evident when the inhibition *rate* is measured as a function of the competing substrate concentration.

Two forms of the membrane-bound or isolated Complex I were identified in our previous studies on the pre-steady-state kinetics of the rotenone-sensitive reactions catalyzed by the enzyme [34] and the thermally induced de-activation was originally analyzed in terms of irreversible kinetics [31,33]. More close examination reported here showed that an equilibrium between those forms is established. It is not surprising that the equilibrium was overlooked because the residual rotenone-sensitive activity of NADH oxidase is only a small fraction of the original activity (Figs. 6 and 7). The equilibrium between two forms may have two important consequences. First one is concerned with the physiologically significant control mechanism of the reducing equivalents entry point to the respiratory chain. Although the equilibrium is temperature independent, it is expected that the *rate* of equilibration at physiological temperature is high and the steady-state electron flow would controlled not only by the $NADH/NAD^+$ and Q/QH_2 ratios but also by the active/inactive enzyme ratio. The second point worth mentioning is that a substantial fraction of free energy available from the reaction: $NADH + Q \rightarrow NAD^+ + QH_2$ is coupled with the enzyme inactive → active transition in the 'activating' turnover, thus it appears that free energy of the enzyme-catalyzed reaction is partially used to maintain the catalytically competent enzyme conformation. In other words NADH-ubiquinone reductase is an interesting example of the energy-transducting machinery which consumes an energy thus being not a 'perfect' catalyst. Since a dramatic difference in the affinity of the active and de-activated forms to rotenone is evident it seems likely that rotenone clamps the 'non-equilibrium' pulsed state, and an extremely tight binding of rotenone suggests that the enzyme-inhibitor complex is likely falls into a transition-state analogue category [45].

We were unable to demonstrate any $\Delta\bar{\mu}_{H^+}$-dependency of rotenone affinity to NADH oxidase inhibitory site (Fig. 2B). However, much lower than NADH oxidase sensitivity of the reverse electron transfer to the inhibitor was confirmed. Kotlyar and Gutman have offered an explanation for different sensitivity of the direct and reverse reactions which was based on two assumptions: first, that the affinity of rotenone is $\Delta\bar{\mu}_{H^+}$-dependent; second, that two populations of the submitochondrial particles exist and only 'coupled' particles catalyze the reverse reaction whereas NADH oxidase is catalyzed by 'uncoupled' particles [38]. Besides the lack of experimental evidence for functional heterogeneity of our tightly coupled submitochondrial particles preparations, the second assumption is hard to accept on the quantitative basis. The preparations routinely used in our laboratory show an average respiratory control ratio of 8 with NADH as the substrate. The specific activity in the reverse electron transfer is about one-fourth of that in the uncoupled NADH oxidase assay [31]. If only a small fraction of the particles ($(1:8) \cdot 100 = \sim 12\%$) catalyzes the reverse electron transfer the enzyme

turnover number in the reverse electron transfer reaction should be much higher than that in NADH oxidase. Such a conclusion is hard to accept either kinetically and thermodynamically or physiologically. Moreover it is hard if not impossible to explain quantitatively the aerobic succinate-supported reverse electron transfer with the assumption on a small fraction of the particles involved in $\Delta\overline{\mu}_{H^+}$-dependent reactions. To explain different sensitivity of Complex I to rotenone in the ubiquinone reductase and reverse electron transfer reactions we propose that different binding sites for oxidized and reduced ubiquinone with different affinity to rotenone are operating in either reactions. In other words the precise mechanisms of the direct and reverse reactions catalyzed by Complex I are not the same. Such an apparent violation of the microreversibility principle appears to hold for other enzymes of the energy-transducing membrane [46,47]. The experimental data which support this hypothesis will be published elsewhere.

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